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A Novel Mutation That Leads to a Congenital Factor XI Deficiency in a Japanese Family

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We have identified a novel mutation leading to a congenital deficiency of the coagulation factor XI (FXI) in a Japanese family. A propositus was a 42-year-old female patient without bleeding tendency. Coagulant activity and the antigen level of FXI in her plasma were below the detectable range. The nucleotide sequences of the FXI gene of this patient were determined by a direct sequence method established in this study. A novel nonsense mutation (CAA; Gly263 → TAA; stop) was identified in exon 8 of the FXI gene. Her parents are first cousins, and a polymerase chain reaction–restriction-fragment length polymorphism analysis revealed that her parents were heterozygous at this nucleotide position. This patient inherited mutant alleles from her parents and is homozygous at this nucleotide position. The nonsense mutation in the FXI gene is responsible for her deficiency of FXI. Am. J. Hematol. 63:165–169, 2000. © 2000 Wiley-Liss, Inc.

Key words: CRM$^-$; nonsense mutation; PCR-RFLP; direct-sequencing

INTRODUCTION

Coagulation factor XI (FXI) is a zymogen of a serine protease family that is involved in the contact phase of the intrinsic pathway of blood coagulation, finally leading to the activation of FIX [1,2]. FXI circulates in peripheral blood as a disulfide bond-linked homodimer (molecular mass 160 kDa) that is complexed with high molecular weight kininogen. The human FXI gene consists of 15 exons distributed over a 23-kb length [3] and is located on the distal end of the long arm of chromosome 4 (4q35) [4].

A congenital deficiency of FXI results in a mild bleeding disorder. Spontaneous bleeding is rare and hemorrhage usually occurs only after trauma or surgery [5]. This disorder is inherited as an autosomal, incompletely recessive trait and is one of the most frequent congenital disorders among Ashkenazi Jews [6]. Homozygotes exhibit a severe deficiency (0–20% of normal plasma FXI activity), while heterozygotes exhibit only a partial deficiency (30–70%) [7,8]. The majority of the patients with FXI deficiency are associated with a lack of the protein in plasma (CRM$^-$) except for a few cross-reacting material-positive (CRM$^+$) cases [9,10]. One of the characteristic clinical features of this disorder is no significant difference in FXI clotting activity between bleeders and nonbleeders [5].

Eighteen causative mutations of congenital FXI deficiencies have been reported so far [11–15], including three major point mutations: a splice junction mutation in intron N, a nonsense mutation in exon 5, and a missense mutation in exon 9 [16]. In this report we established the modified method of sequencing, which reduced the effort required to identify point mutations and found a novel point mutation that was identified in a Japanese patient with CRM$^-$ FXI deficiency.

MATERIALS AND METHODS

Blood Sampling and Coagulation Assays

Peripheral blood was obtained from the patient and her family members with their informed consent. The plasma FXI activity was measured by photometric determination of modified partial thromboplastin time (APTT) using chromogenic substrates as previously described [17]. In brief, 90 μL of 1 mM chromogenic substrates, H-D-Phe Pip-Arg-pNA (Sysmex, Tokyo, Japan), 100 μL of citrated plasma (diluted to 10-fold) from the patient or her family members, 100 μL of citrated plasma lacking FXI (George King Biomedical, Overland, KS), 100 μL of 20

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TABLE I. Primers for Amplification of the Factor XI Gene*

Exon	Sense primers	Antisense primers
1 + 2	TGTAAAACGACGGCCAGTGACATACTAAGATTAACGAC	CAGGAAACAGCTATGACCTTTCCCTCTCCCAGCCCATA
3	TGTAAAACGACGGCCAGTCAACATAACGCATGCCATGTAC	CAGGAAACAGCTATGACCGTCTCCTCGATGTAGAAACAT
4	TGTAAAACGACGGCCAGTCTGTGTGCTGACTTTTAAGAT	CAGGAAACAGCTATGACCTGGTATTTGTTGATTCTGAGA
5	TGTAAAACGACGGCCAGTATCTGGAAGGTACTCATGTC	CAGGAAACAGCTATGACCATCGACCACTCGAATGTCCTG
6 + 7	TGTAAAACGACGGCCAGTTGCAGTTGGAAGAATAAGACAC	CAGGAAACAGCTATGACCTATCCTTACTTGTTAACTCCTTAC
8	TGTAAAACGACGGCCAGTTTACTTTCTCTAGGTGCTGT	CAGGAAACAGCTATGACCACTCTCAGCCAGAATGCAGA
9 + 10	TGTAAAACGACGGCCAGTCTCACTCTGACATGTGGT	CAGGAAACAGCTATGACCAGTCTTGATTGTGATGTATGAA
11	TGTAAAACGACGGCCAGTATTGCTTCTGTTGCAGAGTGTA	CAGGAAACAGCTATGACCATAAATGTGTGAAGAAGATGAAC
12	TGTAAAACGACGGCCAGTGAAATTTCTTTCCCTCTGTTG	CAGGAAACAGCTATGACCTCACCATTGGAGACAA
13	TGTAAAACGACGGCCAGTGGATATATTTTGCGTCTCATA	CAGGAAACAGCTATGACCAAGGTTCCGCTCTTCATTTCT
14	TGTAAAACGACGGCCAGTTCCAGCCTGGGCGACAGAA	CAGGAAACAGCTATGACCTTGCATATATTCCATTGGCTAAGA
15	TGTAAAACGACGGCCAGTCTGAGTTGATCTGTGCACC	CAGGAAACAGCTATGACCTACAACGATCATAGAACGGGAG

*Twelve fragments that included all 15 exons and exon/intron boundaries of the factor XI gene were amplified by PCR. Underlined sequences are added for direct sequencing.

mM $CaCl_2$, and 100 μL of actin were mixed in a ice-bath and then incubated for 8 min at 37°C. The reaction was stopped, and the generated pNA was diazotized by adding the following solutions sequentially: 975 μL of 0.04% sodium nitrate, 975 μL of 0.3% ammonium sulfate, and 975 μL of 0.07% *N*-(*l*-naphthyl)ethylenediamine dihydrochloride. Diazotination changed pNA from yellow to pink. Then absorbance at 545 nm was read, and values were calculated by comparing with normal controls (100%).

Enzyme-Linked Immunosorbent Assay (ELISA) for FXI Antigen

The plasma FXI antigen level was determined by an ELISA, using the goat anti-human FXI antibody (IgG) (Cedarlane Laboratories Ltd, Ontario, Canada) as the primary antibody and rabbit anti-goat immunoglobulin conjugated to peroxidase (DAKO, Glostrup, Denmark) as the second antibody.

Fifty microliters of the patient's plasma diluted 1:1, 1:5, and 1:10 with phosphate-buffered saline (PBS), respectively, was added to each well, followed by incubation for 30 min at 37°C and washing twice in PBS. Next, 250 μL of 1% skim milk diluted in 0.1% Tween-20 with PBS (PBS-T) was added to each well, followed by incubation at 4°C for 17 hr. After the plate was washed twice in PBS-T, 100 μL of the primary antibody diluted 1:1000 in PBS-T was added to each well, and incubation was performed at 20°C for 2 hr, followed by two washes in PBS-T. Fifty microliters of the second antibody diluted 1:10,000 in PBS-T was added to each well, followed by incubation at 20°C for 30 min. After the plate was washed three times in PBS-T, 100 μL of 40% O-phenylenediamine dihydrochloride (OPD) solution in PBS-T was applied to each well, followed by incubation for 20 min. The enzymatic reaction was stopped by the addition of 30 μL of 2 M H_2SO_4, and then absorbance at 490 nm was measured using an ELISA reader (Bio-Rad, Hercules, CA).

Polymerase Chain Reaction (PCR) and Direct Sequencing Analysis Using a Modified Method

Genomic DNA was isolated from the peripheral leukocytes as previously described [18]. Fifteen exons of the FXI gene were amplified by PCR using genomic DNAs as templates [19]. To clarify point mutations by direct sequence, we used designed extended primers in which M13 forword or reverse primer sequences were added to the 3′ end of the PCR primers for factor XI exons. The sequences of the primers used for the PCR are listed in Table I. The underlined sequences of the primers were designed and added for direct sequencing. Approximately 200 ng DNA was added to 100 μL of master mix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM of each primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA). The conditions for the PCR were as follows: 94°C for 10 min (one cycle), 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec (40 cycles), and 72°C for 10 min (one cycle). When extended primers were used for PCR, nonspecific primer annealing to template DNA in a first-round denaturing step prevent amplification of target fragments. To overcome this nonspecific annealing, we used AmpliTaq Gold, of which polymerase activity became active after incubation at high temperature. This feature of the enzyme diminished nonspecific amplification dramatically and make it possible to perform PCR using designed extended primers.

The PCR products were electrophoresed in 2% agarose gels containing Tris acetate buffer, stained with ethidium bromide, and photographed.

The PCR products were purified with a PCR purification kit (QIAGEN KK, Tokyo, Japan), and 300 ng of each PCR product was used for direct sequencing as templates. The nucleotide sequences of the PCR products were determined directly using the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit with

21M13 and M13 Reverse dye-primers (Perkin-Elmer) according to the manufacturer's protocol.

PCR–Restriction-Fragment Length Polymorphism (PCR–RFLP) Analysis

The PCR products amplified using the primers for exon 8 (Table I) and genomic DNA from the patient, her family, and normal donors were digested with the restriction enzyme *Acc*I (Takara, Kyoto, Japan). The reaction mixtures were fractionated in 2% agarose gels including 1× Tris boric acid buffer and then stained with ethidium bromide and photographed under ultraviolet light.

RESULTS

Clinical Course and Coagulation Assays

A 42-year-old female was referred to our clinic because her preoperative laboratory examination for a brain tumor (neurinoma) revealed a markedly prolonged APTT value (101.5 sec, control 36.0 sec). Her platelet count (196×10^9/L), bleeding time (2.5 min), prothrombin time (PT) value (11.3 sec, control 11.4 sec), and plasma fibrinogen concentration (250.4 mg/dL) were within normal range. She had no history of excessive bleeding and had given birth to a child without bleeding complication. She had not been transfused with any blood products. None of her family members had suffered from a bleeding tendency. However, it should be noted that her parents were first cousins. The plasma clotting activities of coagulation factors involved in the intrinsic pathway, including factors XII, IX, VIII, V, X, and prothrombin were within normal range. However, the clotting activity of FXI in the patient's plasma was less than 1% of the control level, and FXI antigen could not be detected by ELISA. The assay for FXI clotting activity in the plasma samples of members of the patient's family revealed that her younger brother was deficient and that both parents and an elder sister were partially deficient for FXI (Fig. 3). These results indicated that the prolonged APTT value of the patient was attributable to the congenital FXI deficiency.

After the transfusion of 1200 mL of fresh frozen plasma, the patient's APTT value decreased to 40.0 sec, and the neurinoma operation was performed successfully. The patient did not suffer from any excess bleeding during her hospitalization.

Novel Mutation of the FXI Gene

To identify the causative mutation of this FXI deficiency, we sequenced the FXI gene of the patient. The nucleotide sequences of these 12 DNA fragments were determined by a modified direct dideoxynucleotide cycle sequencing method. The result revealed a novel type of point mutation in the FXI gene. A change of C to T in exon 8 of the gene results in a nonsense mutation in the third apple domain of FXI (Fig. 1); CAA (glycine 263) → TAA (stop). No wild-type sequence was detected at this nucleotide position in this patient. No other changes were detected in the FXI gene of this patient.

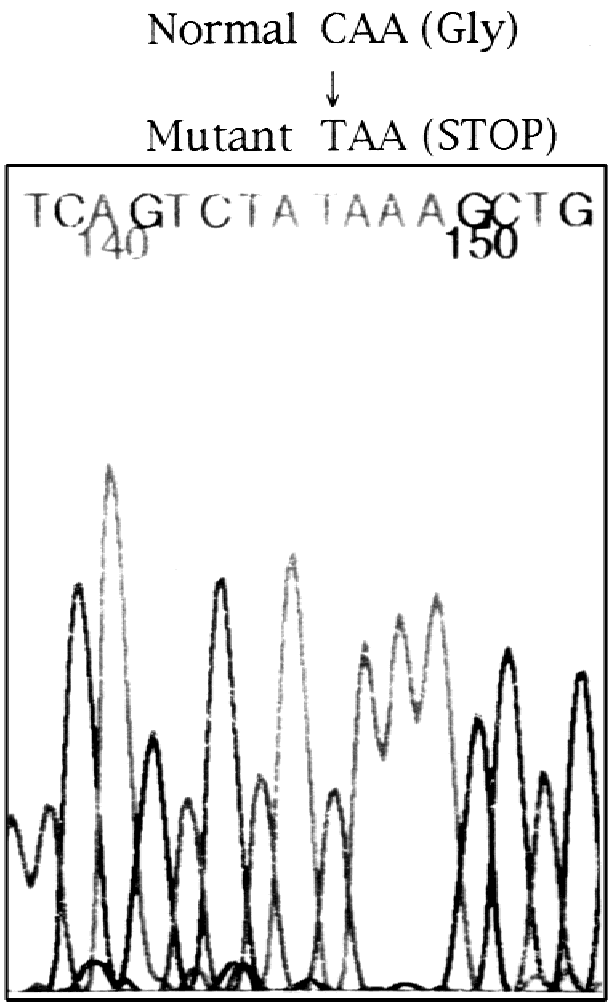

Fig. 1. C to T point mutation in exon 8 of the factor XI gene of the patient. The nucleotide sequences were directly determined by a modified direct sequencing method established in this study. Very low nonspecific signals were detected. This mutation changes CAA (encoding for glycine 263) to TAA for the stop codon. No wild-type sequence was detected.

PCR–RFLP Analysis of the Patient's Family Members

The mutation identified in this patient changes the nucleotide sequence of GTCTC to GTCTT and abolishes an *Acc*I restriction enzyme recognition site. Thus, this mutation can be easily identified by digestion of 221-bp PCR products amplified using primers for exon 8 (Table I) with *Acc*I. Although the PCR product from a normal donor could be cut into two fragments of 160 and 61 bp with this digestion, the PCR products from the patient

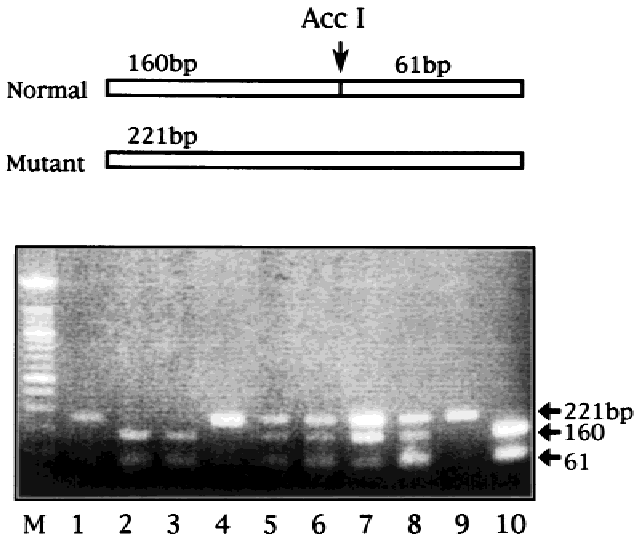

Fig. 2. PCR–RFLP analysis of the patient's family members. Upper: The PCR–RFLP scheme. Normal: PCR products amplified using primers for exon 8 from a normal donor have an *Acc*I site and can be cut into two fragments of 160 and 61 bp. Mutant: PCR products from the patient have no *Acc*I site and cannot be cut. A single band of 221 bp is detected. Lower: Results of the PCR-RFLP analysis. M, DNA molecular weight marker, 100 bp ladder (Boehringer Mannheim, Tokyo, Japan). Lane 1, PCR product before digestion with *Acc*I; lanes 2–10, PCR products digested with *Acc*I. Lane 2, normal donor; lane 3, husband; lane 4, patient; lane 5, father; lane 6, mother; lane 7, sister; lane 8, child; lane 9, brother 1; lane 10, brother 2.

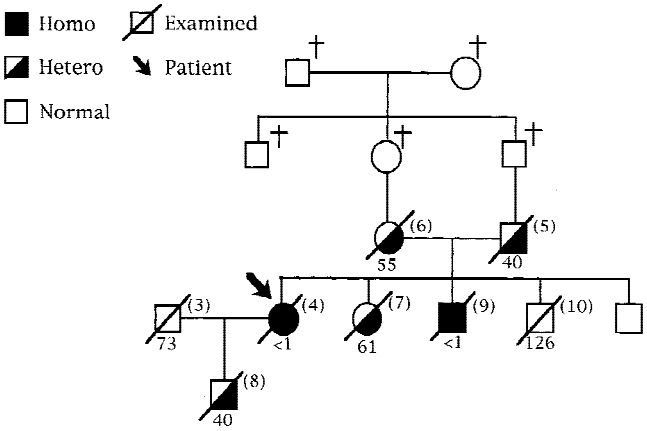

Fig. 3. Pedigree of the patient's family. The symbols indicate genotypes and sex distinction. The factor XI activity in the plasma is given below the symbols (normal range: 65–130%). Numbers in parentheses correspond to the sample numbers in Fig. 2. The patient's parents are first cousins.

could not be cut, and a single band of 221 bp was detected (Fig. 2).

To study the inheritance of this mutation of the FXI gene in the patient's family, we screened her family members by using this PCR–RFLP analysis (Fig. 2). In her parents, elder sister, and son, three bands of 61, 160, and 221 bp were detected, which suggests that these members had both normal and mutant alleles of the FXI gene. They are heterozygous at the nucleotide position. The sequences of the mutated alleles of these family members were determined, and the mutations were identical to that detected in the patient (data not shown). In the patient and her younger brother, only single bands were detected, which suggested that they had only mutated alleles and were homozygous for this mutation (Fig. 3).

DISCUSSION

The best method reported to date for identifying point mutations is direct sequencing. However, one of the problems with the direct sequencing of PCR products is the appearance of nonspecific signals.

Direct sequencing with dye-primers shows low nonspecific signals. We used extended designed PCR primers in which artificial sequences are added to the 3′ end of primers for amplification of the target gene. It is difficult to perform PCR using designed primers, since nonspecific annealing prevents the amplification of a target gene. To overcome this difficulty, we employed a new thermostable DNA polymerase, AmpliTaq Gold. This new polymerase reduced the nonspecific annealing and made it possible to perform the PCR using designed primers. The PCR products amplified by this method could then be sequenced directly using commercially available dye-primers, the sequence of which are identical to artificial sequences added to the each primer. This technique dramatically reduced the background of the data and saved time to identify the mutation.

In the present study, we identified the novel point mutation in the FXI gene that results in a congenital FXI deficiency (CRM^-) in a non-Jewish family. This point mutation in exon 8 leads to a nonsense mutation in the third apple domain of FXI: CAA (glycine 263) to TAA (stop).

Eighteen causative mutations of the congenital FXI deficiency have been reported: three nonsense mutations, ten missense mutations, and five splicing site abnormalities. Three nonsense mutations are located in exons 5 and 7, which truncate the proteins in the middle of the second and third apple domains, respectively.

Except for a few CRM^+ cases, the majority of patients with FXI deficiency exhibit a lack of the protein in the plasma (CRM^-). A premature termination codon may result in a decrease or instability of mRNA or the generation of truncated unstable molecules that may be degraded intracellularly without secretion. These changes lead to the deficiency of FXI in plasma [20–22]. In an experimental model, mRNAs with nonsense mutations near the 5′ terminus have been found to be much more

unstable than mRNAs with nonsense mutations near the 3′ ends [23,24]. Since the nonsense mutation occurs near the 5′ terminus in the coding region of the FXI in the present patient, it seems more likely that a decrease in the amount and/or stability of the mRNA contributes to the CRM^- phenotype.

With a PCR–RFLP analysis using an *Acc*I, we screened the family members of this patient. Her parents have both wild-type and mutant alleles. Since her parents are related to each other, their mutant alleles may come from an identical origin. The patient and her brother exhibited only mutated alleles, indicating that they inherited mutated alleles from their parents.

In this family, the presence of this point mutation is associated with a decrease of FXI in the plasma; the homozygotes for this mutation exhibited the absence of FXI, while the heterozygotes showed a moderate deficiency. Accordingly, we concluded that a novel nonsense mutation in exon 8 of the FXI gene is responsible for the congenital FXI deficiency.

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